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EVALUATION OF TWO METHODS FOR MEASURING NONSPECIFIC IMMUNITY IN TIGER SALAMANDERS (*AMBYSTOMA TIGRINUM*)

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ABSTRACT: Study of amphibian immunotoxicology is a growing area of research, but very little information is available on how environmental contaminants affect disease resistance in urodele amphibians. Urodele amphibians lack the more highly evolved aspects of the specific immune system that are present in anurans, birds, and mammals. Instead, these animals rely more heavily on innate defense mechanisms than do anurans to provide rapid, nonspecific protection from pathogens. Thus, it is prudent that immunotoxicologic research with urodele amphibians includes an evaluation of effects of contaminant exposure on nonspecific immunity. The objectives of this study were to measure the phagocytic and oxidative-burst activity of peritoneal neutrophils collected from a urodele, the tiger salamander (*Ambystoma tigrinum*), and to evaluate the use of these assays in immunotoxicologic research using urodele amphibians. Using tiger salamanders collected in August 2000, phagocytosis and oxidative-burst assays modified from mammalian protocols were conducted through October 2001. Results indicated that large numbers of peritoneal neutrophils for use in immunotoxicologic tests can be collected from salamanders injected with thioglycollate. Moreover, these neutrophils readily engulfed foreign material (phagocytic activity) and produced measurable amounts of hydrogen peroxide (oxidative-burst activity). Phagocytosis was effectively inhibited by incubating cells with sodium azide ($P<0.001$), and quantification of phagocytosis using flow cytometry was well correlated with manual counts ($r=0.84$, $P<0.001$). Dexamethasone treatment reduced phagocytic activity as measured by manual counts ($P<0.02$), suggesting that this test is useful for detecting alteration by immunosuppressive agents. In contrast, oxidative function was unaffected by dexamethasone treatment, and results from the oxidative-burst assay were generally less consistent than those from the phagocytosis assay. Based on these results, phagocytic activity of peritoneal neutrophils may be a useful endpoint in immunotoxicologic studies to evaluate the impact of environmental contaminants on innate defense mechanisms in urodele amphibians.

Key words: *Ambystoma tigrinum,* bioindicator, immune response, immunotoxicology, oxidative burst, phagocytosis, tiger salamander.

INTRODUCTION

Tiger salamanders (*Ambystoma tigrinum*) are the most widely distributed salamander species in North America (Petranka, 1998), but some populations have experienced mass mortality as a result of recent outbreaks of infectious disease. In particular, *Ambystoma tigrinum* virus (Jancovich et al., 1997) and *Regina ranavirus* (Bollinger et al., 1999) have been identified as highly infectious and virulent iridoviruses responsible for high mortality among tiger salamanders. Infectious diseases have been identified as the proximate cause of death in other amphibian population die-offs (Carey et al., 1999), and such diseases are playing an important role in the recent, global declines in amphibian populations (Corn, 2000). Although some investigators have supported the idea that contaminant-induced immune alteration has decreased host resistance in amphibian populations (Carey, 1993), this hypothesis remains an open, and as yet unaddressed, question in the wild.

Amphibians are increasingly used as bioindicators of environmental health, so it is important to develop methods for evaluating the effects of environmental contaminants on these species. Amphibian immunotoxicology, which examines how environmental contaminants affect amphibian immune systems, is an important area of research within the field of amphibian ecotoxicology (Sparling et al.,

2000). Although there has been recent work on anuran immunotoxicology (Taylor et al., 1999; Christin et al., 2003; Gilbertson et al., 2003), very little is known about the effects of environmental contaminants on urodele immune systems. It is important to distinguish between these two amphibian taxa because the immune system of urodele amphibians, such as tiger salamanders, is structurally different and appears to be less responsive than either anuran or mammalian systems (Kaufman and Volk, 1994). Although they have managed to survive pathogenic and environmental challenges for eons, urodele amphibians lack some of the more evolved aspects of lymphocyte-mediated, specific immunity that are present in anurans, birds, and mammals. Thus, it is not practical for researchers working with urodele amphibians to use many of the classic, immunotoxicologic techniques that assess specific immunity, such as the phytohemagglutinin skin test of T-cell function and the rosette assay for B-cell antibody response (Froese, 2002).

Urodele amphibians rely heavily on innate defense mechanisms to provide rapid, nonspecific protection from pathogens. Because of their importance in preventing disease, these mechanisms of nonspecific immunity should be evaluated in immunotoxicologic research with urodele amphibians. The innate defense mechanisms of urodele and anuran amphibians include antimicrobial peptides, complement, natural killer cells, and phagocytic cells (Carey et al., 1999). Phagocytic cells, such as macrophages and neutrophils, recognize, engulf, and destroy pathogenic organisms and other foreign material. The phagocytosis functional assay can be used to evaluate the ability of phagocytic cells to recognize and engulf foreign material. The ability of these phagocytic cells to subsequently destroy material once it has been engulfed can be evaluated with the oxidative-burst assay, which measures the production of reactive oxygen species. There are published protocols that describe methods for conducting these two assays using cells from invertebrates, fish, birds, and mammals (Brousseau et al., 1999), but testing in urodele amphibians has not been well described. One publication by Johnson and colleagues (2000) described methods for phagocytosis and oxidative-burst assays using splenic macrophages from tiger salamanders.

We modified published protocols and investigated the alternative possibility of using peritoneal neutrophils in the phagocytosis and oxidative-burst assays. Our objective was to measure the phagocytic and oxidative-burst activity of peritoneal neutrophils collected from tiger salamanders and to validate the methods used. We chose to examine neutrophils because those cells act against foreign material more quickly than do macrophages and are thus considered to be a first line of defense (Tizard, 2000). To determine the value of using these assays in immunotoxicologic research with urodele amphibians, we evaluated whether the phagocytosis and oxidative-burst assays were significantly affected by exposure to a known immunosuppressive agent, thus establishing toxicologic relevance. The methods followed here can be conducted using nonlethal techniques, which decrease biological costs while potentially increasing information collected from individuals.

MATERIALS AND METHODS

Study animals

Late-larval and recently metamorphosed tiger salamanders were collected by seine netting agricultural dugouts and other small water bodies near Saskatoon, Saskatchewan, Canada (52°07′, 106°38′) during August 2000. The animals were housed in glass aquaria (74×30×36 cm) in a room maintained at 20–22 C with ambient or 12-hr light:12-hr dark conditions. Animals were acclimated for at least 2 wk before being used in experiments, which continued through October 2001. They were fed three times weekly with a mixed diet of live mealworms (*Tenebrio mollitor*) or superworms (*Zophobas morio*), and neonatal, laboratory mouse carcasses. Individual animals were identified by the unique markings on the dorsal surface of

their heads. The use of animals in this research was approved by the University Committee on Animal Care and Supply and was in accordance with the Canadian Council on Animal Care guidelines.

Collection of neutrophils

Intraperitoneal (i.p.) injection of thioglycollate medium 3 to 4 days before cell collection can significantly increase the number of phagocytic cells collected from the peritoneal cavity (Gammie and Ruben, 1986). In the present study, 27 (20 control, seven dexamethasone-treated) tiger salamanders were injected i.p. with 0.8 ml of sterile Brewer modified-thioglycollate medium (Becton-Dickinson, Franklin Lakes, New Jersey, USA) at 96 and 24 hr before the assays. Collection of peritoneal cells was adapted from the procedure described by Plytycz and colleagues (1986) for removing peritoneal macrophages from frogs. Salamanders were anesthetized by topical application of 20% benzocaine gel (Oragel®, Dal Pharmaceutics Canada, Inc., Barrie, Ontario, Canada) to the dorsal surface of the head and neck. A 20-gauge, intravenous catheter was inserted into the peritoneal cavity, and 5 ml of amphibian phosphate buffered saline (APBS) containing 10 U/ml heparin sodium salt (Gibco BRL,® Burlington, Ontario, Canada) was instilled into the peritoneal cavity to suspend the cells. The lavage fluid containing suspended cells was drawn out through the catheter and transferred to a sterile 15-ml polypropylene conical centrifuge tube on ice. Lavage fluid was centrifuged at 200 × G for 10 min at 4 C, and pelleted cells were suspended in cold APBS. Cell viability was assessed using trypan-blue exclusion, and neutrophil concentration was adjusted to 0.5 to 1.0×10^6 cells/ml. This cell suspension was subsequently used for the phagocytosis and oxidative-burst assays. Smears of this cell suspension from three control animals were stained with Wright's-Giemsa stain to confirm the composition of the peritoneal cell population.

To examine the effects of thioglycollate injection on circulating leukocyte populations, air-dried smears of blood collected from the heart or caudal vein were stained with Wright's-Giemsa stain. Total and differential counts of circulating white blood cells were estimated using the procedure described by Lane (1996), and absolute numbers of white blood cells from tiger salamanders injected with thioglycollate ($n=19$) were compared with those from animals that had not been injected ($n=19$). Results were compared using *t*-tests for normally distributed data or Mann-Whitney rank-sum tests when the data were not normally distributed.

Phagocytosis assay

The phagocytosis functional assay was adapted from a protocol for murine peritoneal macrophages described by Brousseau and colleagues (1999). Briefly, phagocytic neutrophils were incubated with fluorescent microspheres, and the number of microspheres engulfed by each neutrophil was measured by flow cytometry. The culture medium, incubation temperature, and incubation time were modified to adapt the mammalian protocol to amphibian cells. The mammalian protocol uses supplemented RPMI 1640 medium and samples are incubated at 37 C for up to 90 min (Brousseau et al., 1999). In comparison, our pilot study confirmed that optimal results with tiger salamander cells resulted from using complete amphibian L-15 culture medium with incubation at 27 C for 18 hr. Amphibian L-15 was prepared by diluting Leibovitz's L-15 medium (Gibco) 2:1 with distilled water, adding HEPES buffer solution (Gibco) to a final concentration of 12.5 mM, and adjusting the pH to 7.5–7.6. Complete amphibian L-15 also contains 10-mM $NaHCO_3$ (BDH®, Toronto, Ontario, Canada), 50-μM 2-mercaptoethanol (Sigma-Aldrich, Oakville, Ontario, Canada), 100-U penicillin/ml, 100-μg streptomycin/ml (Gibco), and 0.25% bovine serum albumin, fraction V (BSA; Sigma-Aldrich; Koniski and Cohen, 1992).

The salamander phagocytosis assay ($n=13$) was performed by placing 0.5 ml of neutrophil cell suspension containing 0.5 to 1.0×10^6 cells/ml into each of two 15-ml polypropylene conical-centrifuge tubes on ice. The cell suspensions were centrifuged at 200 × G for 10 min at 4 C, and the supernatant was discarded. One pellet was suspended in complete amphibian L-15, and the other pellet in the same media plus 0.2% w/v sodium azide (BDH). Sodium azide inhibits phagocytosis, so the fluorescence associated with these negative control cells was the result of adherence of microspheres to the surface of the cell membrane (Brousseau et al., 1999). The effect of sodium-azide treatment on phagocytic function was evaluated using paired *t*-tests.

All tubes were incubated for 1 hr in a humidified incubator at 27 C with 5% CO_2. Yellow-green fluorescent carboxylate-modified microspheres (0.2-μm diameter; Molecular Probes, Eugene, Oregon, USA) were added to each cell suspension at a ratio of 100:1 microspheres to cells, and incubation continued for an additional 18 hr with gentle mixing. At the end of the incubation period, the cell suspen-

sions were layered over a centrifugation gradient of 3% w/v BSA in dilute L-15 and centrifuged at 4 C for 8 min at 150 × G. The supernatant containing free microspheres was aspirated, and 0.5 ml of 0.5% formalin (BDH) in IsoFlow® (Beckman Coulter, Mississauga, Ontario, Canada) was added to each tube to fix the pelleted cells. The fixed cells were stored in the dark at 4 C for up to 24 hr until samples were acquired and analyzed by flow cytometry (Epics Elite ESP®, Beckman Coulter).

For each sample, 10,000 events were acquired at a rate of 100 to 200 events per second. The flow cytometer PMT2 parameter measured wavelengths of 488 to 525 nm, which correlated well with the microspheres' excitation and emission wavelengths of 490 and 515 nm, respectively. Cells that had not been incubated with microspheres were used to adjust the gates for acquisition and analysis. These gates, along with the forward scatter discriminator, excluded cellular debris and free microspheres, but it was not possible to successfully gate out nucleated erythrocytes which contaminated some of the samples. The fluorescence histograms produced by the flow cytometer were used to calculate the percentage of gated cells with one or more microspheres, the percentage of gated cells with three or more microspheres, and the mean number of engulfed microspheres per gated cell. This last parameter was calculated using the following equation from Brousseau et al. (1999):

$$\text{Mean number of microspheres per cell} = \frac{(\%\ A1^+)(\text{Fl } A1^+)}{(\text{Fl } A1)} - \frac{(\%\ C1^+)(\text{Fl } C1^+)}{(\text{Fl } C1)}$$

% $A1^+$ = percentage of gated cells from untreated sample with one or more microspheres

Fl $A1^+$ = mean fluorescence of cells from untreated sample with one or more microspheres

Fl A1 = mean fluorescence of cells from untreated sample with one microsphere

% $C1^+$ = percentage of gated cells from sodium azide-treated sample with one or more microspheres

Fl $C1^+$ = mean fluorescence of cells from sodium azide-treated sample with one or more microspheres

Fl C1 = mean fluorescence of cells from sodium-azide treated sample with one microsphere

Use of the flow cytometer for quantifying phagocytic activity was validated by examining wet-mounts of the final cell suspensions using light microscopy. For this manual count, 200 cells per slide were examined at 400× magnification to determine the number of engulfed microspheres. The results of the manual count were expressed as the percentage of cells with one or more microspheres and the percentage of cells with three or more microspheres. The results from flow cytometry and manual counts were compared using Pearson product moment correlation. Although the number of cells examined using flow cytometry was much higher, we feel that the manual counts were more accurate because we were able to identify neutrophils and include only those cells in the manual counts. In contrast, the flow cytometer may have included other cell types and clumps of debris in the total cell count, thus compromising the accuracy of those results.

Oxidative-burst assay

A fluorescent, microplate assay for detecting oxidative products of mammalian phagocytic cells (Rosenkranz et al., 1992) was adapted for use in tiger salamanders. The assay relies on the oxidation of 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) to the fluorescent product dichlorofluorescein (DCF). The amount of fluorescence is indicative of the amount of hydrogen peroxide produced. The mammalian assay was modified for use with amphibian cells by decreasing the incubation temperature from 37 to 27 C and changing the culture medium from phosphate-buffered saline with calcium and magnesium to APBS. Other published protocols (Rosenkranz et al., 1992; Johnson et al., 2000) recommend adding 0.1 nM–1.0 μM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) to the culture medium to stimulate hydrogen peroxide production. In initial trials with cells from tiger salamanders stimulated with i.p. thioglycollate, PMA with final concentrations up to 100 nM did not increase the cellular production of hydrogen peroxide. This step was therefore omitted from subsequent assays.

The oxidative-burst assay tested on 27 tiger salamanders (but we used the results from 18 only: 13 control and five dexamethasone treated, because the other samples were contaminated with red blood cells) was performed by dispensing peritoneal cell suspensions at 0.5 to 1.0×10^6 cells/ml of APBS into black cliniplates (Thermo Labsystems, Franklin, Massachusetts, USA) at 180 μl per well. A final concentration of 50-μM DCFH-DA was achieved by adding 20 μl of the working solution (500 μM in APBS) to each well. All plates were incubated at 27 C for 90 min, and fluorescence was mea-

sured immediately using a MFX Microtitre® Plate Fluorometer (Dynex Technologies Inc., Chantilly, Virginia, USA), with excitation and emission wavelengths of 485 nm and 538 nm, respectively. Results were expressed as relative fluorescence units (RFU).

Dexamethasone study

The immunosuppressive action of dexamethasone has been described in *Xenopus* (Saad, 1988), but its effect in urodeles is not yet described. The potential effect of dexamethasone on the phagocytic and oxidative-burst activity of salamander peritoneal neutrophils was evaluated in an attempt to assess the relative sensitivity of these measures of innate immune function. This chemical was examined for use as a positive control for immunosuppression in tiger salamanders. The effects of dexamethasone on mammalian neutrophils are not completely understood but may include inhibition of chemotaxis (i.e., movement toward certain chemicals present at sites of infection or tissue damage) and intracellular production of reactive microbicidal compounds (Bach, 1975). Tiger salamanders were randomly assigned to control (n=20) or dexamethasone (n=7) treatment groups. The number of animals in the dexamethasone group was dictated by the total number available. Salamanders in the dexamethasone treatment group received 0.1-mg/kg dexamethasone (Vetoquinol, Lavaltrie, Quebec, Canada) by intramuscular injection weekly for 4 wk. All animals were euthanized 3 or 4 days after their final dexamethasone injection, and peritoneal cells were collected for phagocytosis and oxidative-burst assays. Results from the control and dexamethasone-treated groups were compared using a two-tailed, independent-samples t-test after checking the data for normality and equality of variance. All statistical analyses were done using SigmaStat® 2.03 statistical software (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Collection of neutrophils

The viability of peritoneal cells incubated at 20 to 26 C for 18 hr was 95±7% (n=8). Neutrophils were the most abundant cell type in the peritoneal lavage fluid collected from tiger salamanders injected with thioglycollate. The composition of the peritoneal cell population (mean±SD) was 85±3% neutrophils, 11±5% lymphocytes, 2±2% monocytes, and 2±2% basophils (n=3). In addition to promoting the movement of neutrophils into the peritoneal cavity, i.p. injection of thioglycollate medium also affected circulating leukocyte populations. Salamanders injected with thioglycollate (n=19) medium had fewer lymphocytes (P=0.023), fewer monocytes (P<0.001), and more neutrophils (P=0.008) in peripheral circulation than salamanders that had not been injected with thioglycollate (n=19).

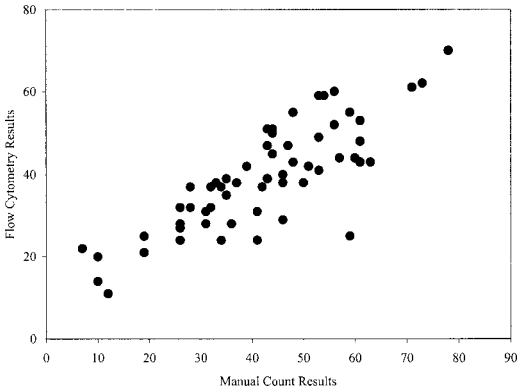

FIGURE 1. Association between flow-cytometry and manual-count results for the percentage of tiger salamander peritoneal neutrophils with three or more engulfed microspheres in samples with minimal erythrocyte contamination after 18 hr of incubation. Results were analyzed with Pearson product moment correlation (r=0.837; P<0.001; n=13).

Phagocytosis assay

The results from flow cytometry and manual counts were well correlated (r=0.837; P<0.001; n=13) when there was minimal contamination of the lavage fluid with nucleated erythrocytes (Fig. 1). However, in cases with moderate to high levels of erythrocyte contamination, the results were not well correlated. Consequently, samples with substantial erythrocyte contamination were excluded from analysis. This resulted in smaller, final sample sizes for the control and dexamethasone-treated groups of 13 and five, respectively.

Results from both the flow cytometer and the manual counts showed that treatment with sodium azide successfully inhibited phagocytic activity of peritoneal neutrophils. For example, manual counts of cells collected from the control animals

from the dexamethasone study revealed that 50±12% of untreated cells engulfed three or more microspheres, but only 11±8% of cells treated with sodium azide were associated with three or more microspheres ($P<0.001$).

Treatment with dexamethasone reduced the percentage of peritoneal neutrophils engulfing one or more microspheres ($P=0.024$) and three or more microspheres ($P=0.010$), as evaluated by manual counts (Fig. 2a). One or more microspheres were engulfed by 68±11% of the cells from control animals and by 52±16% of those from dexamethasone-treated salamanders, whereas three or more microspheres were engulfed by 50±12% of the cells from control animals and by 30±15% of those from dexamethasone-treated salamanders. Although the flow cytometry results showed similar trends (Fig. 2b), the differences were not statistically significant ($P>0.05$). One or more microspheres were engulfed by 65±8% and 56±13% of cells from control and dexamethasone-treated salamanders, respectively, whereas 45±10% and 35±13% of cells engulfed three or more microspheres. Dexamethasone treatment did not affect the mean number of microspheres per gated cell ($P=0.35$). Cells from control animals had, on average, 6.5±2.2 microspheres per gated cell, whereas those from the dexamethasone-treated animals had 5.3±2.9 microspheres per gated cell.

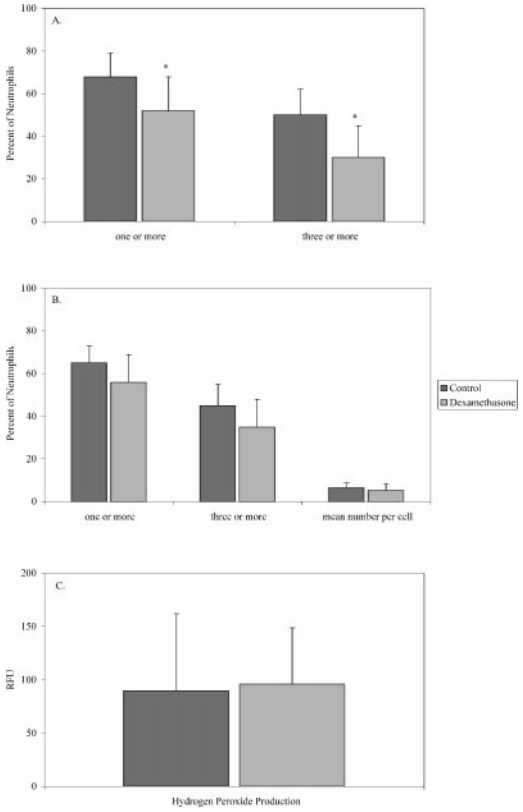

FIGURE 2. Effects of dexamethasone treatment on phagocytic and oxidative-burst activity of tiger salamander peritoneal neutrophils. (a). Percentage of cells with one or more microspheres and three or more microspheres as determined by manual counts. (b). Percentage of cells with one or more microspheres, the percentage with three or more microspheres, and the mean number of microspheres per cell as determined by flow cytometry. (c). Production of hydrogen peroxide. RFU=relative fluorescence unit. Asterisk denotes a significant difference between control and dexamethasone-treated salamanders as determined by two-tailed independent samples *t*-tests ($P<0.05$).

Oxidative-burst assay

Dexamethasone treatment did not alter the ability of salamander peritoneal neutrophils to produce hydrogen peroxide ($P=0.87$; Fig. 2c). When the analysis was limited to samples with minimal erythrocyte contamination, the fluorescence production for cells from the control animals ($n=13$) averaged 90±72 RFU, whereas cells from the dexamethasone-treated animals ($n=5$) produced 96±53 RFU.

DISCUSSION

The functional phagocytosis assay described by Brousseau and colleagues (1999) was successfully adapted for use with tiger salamander neutrophils. The most critical changes to standard mammalian methodology included using complete amphibian L-15 as the cell culture medium in place of supplemented RPMI 1640, incubating at 27 C compared with 37 C, and incubating with microspheres for 18 hr instead of 90 min. Johnson and colleagues (2000) used a similar method to characterize the phagocytic activity of

splenic macrophages from tiger salamanders. One of the important differences between our method and the one described by Johnson and colleagues is that we used sodium azide to inhibit phagocytosis instead of gadolinium (III) chloride. In addition, we used peritoneal neutrophils, whereas the previous study involved splenic macrophages. To our knowledge, this is the first report describing the phagocytic activity of granulocytes from urodele amphibians. Because neutrophils act more quickly than macrophages (Tizard, 2000), it is important to develop the tools necessary to examine effects of immunosuppressive agents on neutrophil activity. Moreover, the methods described in this paper for collecting peritoneal neutrophils can be done with the animals surviving, after being temporarily anesthetized. This would have the obvious advantages of reduced biological costs and the potential to conduct follow-up studies on the same animals. Further research could examine whether the phagocytosis assay could be conducted successfully with neutrophils isolated from peripheral blood of urodele amphibians.

Large numbers of peritoneal neutrophils can be successfully collected from tiger salamanders primed (injected) with thioglycollate. Moreover, these cells readily engulf foreign material and produce measurable quantities of hydrogen peroxide. The phagocytosis assay was particularly successful in that treatment with dexamethasone, a known immunosuppressive agent, inhibited phagocytic activity as measured by manual counts. These results suggest that the phagocytosis assay could be used as an endpoint to assess the effects of environmental contaminants on nonspecific, innate defense mechanisms in tiger salamanders. The failure to demonstrate a significant effect of dexamethasone using flow cytometry may have been due to the lower sensitivity of this method relative to microscopic examination.

Although manual counts examine far fewer cells than can be analyzed by the flow cytometer, neutrophils are readily distinguished by an experienced researcher. The flow cytometer probably counts other cell types, clumps of microspheres, and debris as "events," whereas only neutrophils are included in the manual counts. Although flow-cytometry results demonstrated good correlation with the manual counts when there was minimal contamination of the peritoneal lavage samples with erythrocytes, higher degrees of contamination interfered with the accuracy of the flow-cytometer results. In many of the contaminated samples, the flow cytometer tended to show lower percentages of phagocytic neutrophils than were evident from the manual counts, presumably because erythrocytes were being counted as events with no engulfed microspheres. Although we believe that manual counts are more accurate because neutrophils were easily distinguished from other cells types, using flow cytometry may be more practical in studies with larger sample sizes. Additional work is required to optimize the phagocytosis assay for flow-cytometer applications.

To use tiger salamander cells in the fluorescence microplate assay for detecting oxidative products, necessary modifications to the mammalian protocol (Rosenkranz et al., 1992) included changing the culture medium to APBS, reducing the incubation temperature to 27 C, and increasing the total incubation time to 90 min. Johnson and colleagues (2000) recently reported using a flow-cytometric oxidative-burst assay with salamander macrophages. Their methodology involved using supplemented RPMI medium and incubating cells at room temperature for 45 min. In contrast to findings by other researchers (Rosenkranz et al., 1992; Johnson et al., 2000), hydrogen peroxide production in our pilot studies was not enhanced by adding PMA. This lack of response may have been due to the use of thioglycollate to elicit the migration of neutrophils into the peritoneal cavity. The neutrophils may already have been stimulated to produce reactive oxy-

gen species including hydrogen peroxide because of being exposed to thioglycollate so that additional stimulation by PMA was ineffective. This possibility could be examined in additional studies. The observation that dexamethasone treatment did not alter hydrogen peroxide production by salamander neutrophils may indicate that this endpoint is less sensitive than the phagocytosis assay to the effects of certain immunosuppressive agents.

The results of this study with tiger salamanders indicate that the phagocytosis assay is superior to the oxidative-burst assay because it was affected by dexamethasone treatment and the results were less variable. The phagocytosis assay using tiger salamander neutrophils may be a useful research tool in the continued exploration of the possible links between environmental contaminants, immune alteration, and amphibian epizootics. Because urodele amphibians lack the more evolved, specific, immune components present in other taxa, it would be prudent to include an evaluation of innate defense mechanisms in immunotoxicology studies with these animals.

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